Fermentation enhances the content of bioactive compounds in kidney bean extracts

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Abstract

The influence of solid (SSF) or liquid state fermentation (LSF) for 48 and 96 h on the production of water soluble extracts from kidney beans was investigated. SSF was carried out by *Bacillus subtilis*, whilst LSF was performed either by natural fermentation (NF) or by *Lactobacillus plantarum* strain (LPF). SSF extracts showed high soluble phenolic compound content (31-36 mg/g) and antioxidant activity (508-541 μg trolox equivalents/g), whilst LSF extracts exhibited potential antihypertensive activity due to their large γ-aminobutyric acid (GABA) content (6.8-10.6 mg/g) and angiotensin converting enzyme inhibitory (ACEI) activity (>90 %). Therefore, fermentation can be considered as a valuable process to obtain bioactive ingredients from kidney beans, which could encourage their utilization in the formulation of added-value functional foods.

Keywords: kidney beans, fermentation, antioxidants, antihypertensive compounds, functional ingredients.

1. Introduction

Chronic diseases such as cancer, diabetes, obesity and cardiovascular diseases (CVD) represent the leading cause of mortality, accounting for around 36 billion deaths worldwide each year (WHO, 2013). Scientific evidence has demonstrated that diet has an essential role in their prevention and management.

Consumption of traditional fermented legumes has been shown to have protective effects against CVD (Crujeiras, Parra, Abete & Martinez, 2007) and therefore, there is a growing interest to promote their production. Fermentation of legumes brings several advantages since it decreases the non-nutritional factors, improves nutrient digestibility and reduces their allergenicity (Frias, Song, Martinez-Villaluenga, Gonzalez de Mejia, & Vidal-Valverde, 2008; Starzyńska-Janiszewska, Stodolak, & Mickowska, 2013). This technological process also improves the biological activity of legumes, as microbial enzymes bring about the bioconversion of polyphenols into more biologically active compounds (Lee, Lo, & Pan, 2013) and can release bioactive peptides from legume proteins (Martinez-Villaluenga et al., 2012; Torino et al., 2013). Although soybean is the most utilized legume for fermentation, there are other unexplored legumes with a large potential for the production of fermented foods that might contribute to the prevention of CVD.

Solid and liquid state fermentations (SSL and LSF, respectively) are current processing techniques traditionally used to preserve and to enhance the nutritional quality and health promotion properties of legumes (Juan & Chou, 2010; Torino et al., 2013). The type of microorganism involved in the fermentation process plays a key role in the fermentation process. *Bacillus subtilis* has been used as a starter strain for manufacturing soybean fermented products with potential antihypertensive, antithrombotic and fibrinolytic properties (Murakami, Yamanaka, Ohnishi, Fukayama, & Yoshino, 2012; Omura, Hitosugi, Zhu, Ikeda, & Maeda 2005). Recently, Dueñas, Hernández, Robredo, Lamparski, Estrella and Muñoz

(2012) have reported that *B. subtilis* enhance the production of isoflavone aglycones, thus improving the antioxidant activity of fermented soybean.

Lactic acid bacteria (LAB) have also been traditionally used for legume fermentation since they are naturally present in legume grains. There is strong consensus that fermentation of legumes with LAB strains, such as those of *Lactobacillus* genera, can favor the production of bioactive compounds, providing health benefits beyond basic nutrition (Savijoki, Ingmer, & Varmanen, 2006; Frias et al., 2008; Song et al., 2010; Torino et al., 2012). Fermentation of cowpeas with *L. plantarum* led to changes in phenolic composition and improved antioxidant activity (Dueñas, Fernández, Hernández, Estrella, & Muñoz, 2005). It has recently been reported that LAB fermentation of adzuki beans and lentils allows accumulation of γ-aminobutyric acid (GABA), a blood pressure regulator (Liao, Wang, Shyu, Yu, & Ho, 2013; Torino et al., 2013). In addition, soybean milk fermented with LAB exhibited antioxidant, antihypertensive, antiinflammatory (Martinez-Villaluenga et al., 2012) and anti-obesity properties (Lee et al., 2013).

Total world grain bean production increased by 3% in 2005 to 23.5 million tons (FAOSTAT, 2013) and although they are the most important grain legume in direct human consumption in the world (Broughton et al., 2003), their industrial utilization for added-value food ingredients is limited. Therefore, fermentation process represents a strategic approach for diversifying derived bean products to a current market demanding novel functionalfoods with CVD preventive properties.

This work investigated solid and liquid state fermentations in kidney beans for the production of water-soluble extracts with potential antihypertensive effects (angiotensin I converting enzyme inhibitory –ACEI- activity and GABA content), phenolic composition and antioxidant activity. The evolution of microbial populations and pH in fermented beans was also studied. Results derived from this work will provide valuable information on the

composition and bioactivity of kidney bean fermented ingredients in order to develop novel functional foods.

2. Materials and methods

2.1. Seeds

Kidney beans (*Phaseolus vulgaris* var. Pinto) were provided by Semillas Iglesias S.A. (Salamanca, Spain). Seeds were washed with distilled water and dried at 55 °C for 24 h before fermentation.

2.2. Preparation of microbial cultures

Bacillus subtilis CECT 39^T (ATCC 6051) and *Lactobacillus plantarum* CECT 748^T (ATCC 14917) were purchased from the Spanish Type Culture Collection (CECT). Cultures were stored and activated for solid or liquid fermentations as described in Torino et al. (2013).

2.3. Fermentation of kidney beans

2.3.1. Solid state fermentation (SSF)

Cracked beans (100 g) were suspended in sterile distilled water (1:2, w/v) and autoclaved at 121 ºC for 15 min. Sterile cracked seeds (30 g) were homogeneously inoculated with 5 % (v/w) *B. subtilis* (10^5 CFU/g), mixed, aseptically distributed over Petri dishes and incubated for 96 h at 30 ºC and 90 % humidity in a climatic chamber (Snijders-Scientific, Tiburg, Netherlands). SSF samples were withdrawn at 0, 48 and 96 h to determine changes in bacterial populations and pH values. Fermented samples were autoclaved at 121 ºC for 15 min and freeze-dried. SSF water-soluble extracts were prepared by suspending freeze-dried fermented beans in distilled water (1:10, w/v) and kept in continuous agitation for 1 h at room temperature. Extracts were centrifuged at 14534 *g* for 15 min at 10 ºC (Sorval, model RC 6+).

Supernatants were filtered through Whatman nº 1 paper, freeze-dried and stored under vacuum at -20 ºC until further analysis.

2.3.2. Liquid state fermentations (LSF).

Bean flour (200 g) was suspended in sterile distilled water (1:5 w/v). Fermentations were carried out in a 3 L BioFlo/celligen 115 fermenter (New Brunswick Scientific Co., INC, Edison, NJ) either spontaneously by the indigenous microbiota present on seeds (natural fermentation, NF) or with 2 % (v/v) of *L. plantarum* suspension (10^8 CFU/mL) (LPF), at 37 ºC and continuous agitation at 350 rpm for 96 h. Samples were withdrawn at 0, 48 and 96 h to determine changes in bacterial populations and pH. Fermented samples were centrifuged (14534 *g*, 15 min, 6 ºC) and supernatants were freeze-dried and stored under vacuum at -20 ºC for further analysis.

2.4. Microbiological analysis

Fermentations were monitored by withdrawing samples at 0, 48 and 96 h of fermentation using plate counts to determine changes in viable cells. *B. subtilis* were counted in BHI broth supplemented with 1.5 % (w/v) agar, after incubation at 30 ºC for 48 h. LAB were counted in MRS agar after incubation at 37 ºC in a 5 % CO_2 atmosphere for 72 h. Cell counts were expressed as $\log_{10}$ CFU/g for *B. subtilis* and $\log_{10}$ CFU/mL for LAB.

2.5. Chemical analysis

2.5.1. Proteolytic activity

Proteolytic activity in SSF and LSF bean extracts was assessed by measuring the free amino groups following the method reported by Martinez-Villaluenga et al. (2012). Results were expressed as mg peptides/g extract.

2.5.2. GABA content.

The extraction of GABA in SSF and LSF extracts and its quantification by HPLC was carried out as in Torino et al. (2013). Results were expressed in mg GABA/g extract.

2.5.3. Soluble phenolic compounds (SPC)

The content of SPC in bean extracts was determined as in Torino et al., (2013). SPC were quantified by external calibration using gallic acid (Sigma-Aldrich). Results were expressed as mg gallic acid equivalents (GAE)/g extract.

2.5.4. Extraction and analysis of phenolic compounds by HPLC-DAD-ESI/MS

Water-soluble bean extracts were macerated in methanol/trifluoroacetic acid ($1^0/_{000}$):water 80:20 (v/v) for 1 h according to Dueñas, Hernandez, & Estrella (2007) and analysed as follows:

Non anthocyanin phenolic compounds. The extracts were analyzed using a Hewlett-Packard 1100MS chromatograph (Agilent Technologies) equipped with a quaternary pump, diode array detector coupled to an Hewlett Packard Chem Station (rev. A0504) data-processing station, following the method described by Barros, Dueñas, Carvalho, Ferreira, & Santos-Buelga (2012). A Waters Spherisorb S3 ODS-2 C8, 3 μm (4.6 mm×150 mm) column at 35 °C was used. Solvents were (A) 0.1% formic acid in water, and (B) 100% HPLC grade acetonitrile. The elution gradient established was: 15% for 5 min, 15-20% B over 5 min, 20-25% B over 10 min, 25-35% B for 10 min, 35-50% B for 10 min. The flow-rate was 0.5mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths. Mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet was used and detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI (Electrospray Ionization) source, triple

quadrupole-ion trap mass analyzer and controlled by the Analyst 5.1 software. The settings were: zero grade air as the nebulizer gas (30 psi), turbo gas for solvent drying (400 ºC, 40 psi), nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced mass spectrum (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) -45 V, entrance potential (EP) -6 V, collision energy (CE) -10V. Spectra were recorded in negative ion mode between *m/z* 100 and 1000. EPI mode was further performed in order to obtain the fragmentation pattern of the parent ion(s) of the previous experiment using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V.

The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations of different standard compounds. Aldaric, quinic and hexosides derivatives of *p*-coumaric, ferulic and sinapic acids were quantified by the curves of the corresponding free acids; (+)-catechin-hexoside and (+)-catechin-acylhexoside based on the curve of (+)-catechin; flavonols, derivatives of kaempferol, quercetin and isorhamnetin by the curves of kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside and isorhamnetin 3-*O*-rutinoside, respectively. Flavanones, eridictyol derivatives based on the curve of narigenin; and naringenin and hesperetin derivatives as naringenin and hesperitin, respectively. Results were expressed as mg of phenolic compound/g of extract.

Anthocyanins. The extracts were analysed in an AQUA® HPLC system (Phenomenex) equipped with a reverse phase C18 column (5 μm, 150 mm × 4.6 mm i.d) at 35 ºC according to Garcia-Marino, Hernández-Hierro. Rivas-Gonzalo, and Escribano-Bailón (2010). Detection was carried out at 520 nm. MS was performed in the same equipment described above. Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 ºC) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high). Both quadrupole units were set at unit resolution. The ion spray voltage was set at 5000 V in the positive mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively. Setting parameters used for EMS mode were: declustering potential (DP) 41 V, entrance potential (EP) 7.5 V, collision energy (CE) 10 V, and parameters for EPI mode were: DP 41 V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V.

For the quantitative analysis of anthocyanin a calibration curve was obtained by injection of different concentrations of cyanidin 3-*O*-glucoside, malvidin 3-*O*-glucoside and pelargonidin 3-O-glucoside. The results were expressed in μg per g of dry weight (dw).

2.5.5. SDS-PAGE protein profile

Proteins from SSF and LSF extracts were separated by SDS-PAGE under reducing conditions according to Limón, Peñas, Martinez-Villaluenga, and Frias (2014).

2.5.6. Antioxidant activity

Oxygen Radical Absorbance Capacity was determined in the water-soluble bean extracts by fluorescence measurement (ORAC-FL) as described by Torino et al., (2013). Trolox (Sigma) was used as standard. Results were expressed as μg Trolox equivalents (TE)/g extract.

2.5.7. Angiotensin converting enzyme inhibitory activity (ACEI activity)

ACEI activity was determined according to Martínez-Villaluenga et al., (2012). IC_{50} values, defined as the amount of sample that inhibits the activity of ACE by 50%, were determined by sigmoidal dose–response curves in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

2.6. Statistical methods. SSF and LSF processes were independently performed in triplicate. Chemical analyses of each batch were carried out in duplicate, and each determination was analyzed three times. Data were subjected to one-way analysis of variance (ANOVA) using Statgraphics Centurion XVI software, version 16.1.17 (Statistical Graphics Corp, Rockville, MD, USA). Significant differences between samples were determined by using the least significant difference (LSD) test at $P \leq 0.05$ probability level. Principal Components Analysis (PCA) was performed with covariance matrix obtained by data transformation using MultBiplot (Vicente-Villardon, 2010).

3. Results and discussion

3.1. Evolution of pH and microbial growth during fermentation

Changes of pH values during fermentation of beans at the different studied conditions are shown in Table 1. During SSF, pH value significantly increased ($P \leq 0.05$) from 6.3 to 7.0, which could be attributed to the production of ammonia during kidney bean fermentation by *B. subtilis* (Allagheny, Obanu, Campbell-Platt, & Owens, 1996) The population of *B. subtilis* increased sharply during the first 48 h (~3.14 log units) and no further changes were observed. Bacterial growth was reflected in the proteolytic activity, measured as the amount of peptides released during fermentation, which increased significantly from 48 h (5.25 mg/g) to 96 h (29.36 mg/g) (Table 1). The low amount of peptides formed during the first 48 h could be

caused by the difficulty of *B. subtilis* proteases to cleave glycoproteins, phosphoproteins or domains containing a high number of disulfide bridges (Weng & Chen, 2010).

In the course of LSF, pH values gradually decreased significantly ($P \leq 0.05$) for the first 48h, from 6.6 to 4.3 and 3.7 in NF96 and LPF96, respectively (Table 1) due to the production of organic acids by LAB during fermentation (Schindler, Zelena, Krings, Bez, Eisner & Berger, 2012). The initial population of LAB in raw beans was very low (1.01 CFU/mL) and it increased significantly during NF, reaching counts of 8.8 log CFU/mL after 48 h, which remained almost constant to the end of the fermentation (Table 1). LAB counts increased from 7.0 to 9.1 log CFU/mL after 48 h and suffered a significant ($P \leq 0.05$) reduction at the end of fermentation (5.9 log CFU/mL). When colonies from MRS plates were subjected to microscopy analysis, bacilli morphologically corresponding to *L. plantarum* were observed, indicating that the starter added led the fermentation process. The loss of viability of *L. plantarum* after 96 h was previously observed by our group during induced lentil fermentation (Torino et al., 2013). It is known that organic acids released during fermentation exert an inhibitory effect on microbial growth due to an increase in the amount of protons that acidifies the cytoplasm and inhibits many metabolic functions (Lambert & Stratford, 1999). It has been shown that the growth of *L. plantarum* is inhibited when internal pH reach values of 4.6-4.8, corresponding to external cellular pH values of ~3 (McDonald, Fleming, & Hassan, 1990). These findings could explain the loss of viability of *L. plantarum* observed in our study after 96 h (pH 3.7).

LAB counts were consistent with pH values in NF and LPF. Faster acidification was observed in LPF compared to NF due to the higher LAB population. Growth of the LAB population in LSF after 48h was accompanied by a noticeable increase in the proteolytic activity and, as a result, significantly higher concentrations of peptides were found (~160 mg/g extract) (Table 1). However, peptide content was significantly reduced ($P \leq 0.05$) after 96

h in both LSF. This effect could be attributed to the microbial utilization of peptides as a nitrogen source.

3.2. GABA content in bean extracts

GABA contents in water-soluble extracts are also compiled in Table 1. SSF led to a decrease in GABA content (~18%) irrespective of fermentation time. Unlike SSF, NF and LPF processes caused a significant ($P \leq 0.05$) GABA accumulation. The extent of this increase depended on the type of fermentation: NF led to significantly ($P \leq 0.05$) higher GABA levels than LPF (10.6 vs. 9.9 mg/g for NF96 and LPF96, respectively). Similar GABA accumulation was reported in adzuki bean milk fermented with *Lactococcus lactis* and *Lactobacillus rhamnosus* (Liao et al., 2013). These results indicate that effects of fermentation on GABA content are due to the type of microorganism involved in the process. GABA is a non-protein amino acid produced from L-glutamic acid by glutamic acid decarboxylase enzyme (GAD). *B. subtilis* seems to have a weak capacity to produce GABA, mostly when compared with other microorganisms such as LAB (Park & Oh, 2006). These findings are in agreement with the low GABA content observed in SSF bean extracts in this work. In addition, the thermal treatment of cracked seeds during autoclaving used in the SSF process would inactivate endogenous GAD activity from beans, contributing to the reduced GABA concentration observed in SSF extracts. On the contrary, the GABA accumulation observed in LSF (Table 1) suggests that LAB strains stimulate the production of GABA during bean fermentation. It has been previously shown that many LAB strains, including *L. plantarum*, are potential GABA producers (Di Cagno et al., 2010), since they harbor the gene encoding GAD enzyme (Ko, Lin, & Tsai, 2013). It can be postulated that the endogenous bean GAD would be in part responsible for the GABA increase observed during LSF, since previous studies have revealed a rapid plant GAD activation in response to mechanical manipulation, anaerobic conditions

and low pH (Akihiro et al., 2008). The higher GABA content found in NF compared to LPF extracts could be attributed to the higher number of LAB strains producing GAD in NF and also to the different pH of both fermentations. pH seems to play a crucial role in GABA production by LAB. It has been reported that *L. plantarum* DSM19463 synthesized the maximum GABA concentration at pH 6.0 (Di Cagno et al., 2010). The acidic pH observed in LSF in this work could be one of the causes of the decreased GAD activity and, consequently, the low rate of GABA production.

It is well known that GABA has a blood pressure-lowering effect in animals and humans (Inoue et al., 2003) and also acts as a strong secretagogue of insulin from the pancreas, potentially preventing diabetes (Adeghate & Ponery, 2002). Therefore, there is a growing interest to develop GABA-enriched functional foods and fermentation of beans by LAB could constitute an attractive approach to achieve this purpose.

3.3. Content of soluble phenolic compounds in bean extracts (SPC)

Due to the potential positive effects of phenolic compounds and their metabolites, they are the subject of many ongoing research studies. Polyphenols are natural antioxidants, which may protect against multiple chronic diseases (Del Rio, Rodriguez-Mateos, Spencer, Tognolini, Borges, & Crozier, 2013) and legumes are considered a rich source of these compounds. SPC concentration varied in fermented beans depending on the fermentation conditions (Table 1). SSF resulted in a significant ($P \leq 0.05$) increase in SPC after 48 and 96 h (96 and 126 %, respectively), whilst no significant ($P \geq 0.05$) changes or a slight decrease was observed at the end of LSF fermentations. The increase in SPC after SSF could be related to the production of β-glucosidases by *B. subtilis*. This enzyme hydrolyzes β-glucosidic bonds of several phenolic compounds occurring in a conjugated form (phenolic glucosides) to the corresponding phenolic aglycones, increasing the concentration of free polyphenols

(Georgetti, Vicentini, Yokoyama, Borin, Spadaro, & Fonseca, 2009). In fact, the SPC content observed after SSF is within the range of values reported after fermentation of soybean by *B. subtilis* TN51 (Dajanta, Apichartsrangkoon, & Chukeatirote, 2011). Our results indicate that microbial β-glucosidase activity was higher in SSF than in the LSF process. These differences could be attributed to the different fermentation conditions. Sestelo, Poza, and Villa, (2004) reported that the activity of β-glucosidase produced by *L. plantarum* USCI varied greatly depending on the pH in the medium, with the optimal pH range for enzyme activity being from 4.5 to 7.5, while pH values below 4 produced enzyme inactivation. These findings could explain the low modification of SPC content during LSF, due to inactivation of this enzyme at low pH values (3.7). The lack of changes in SPC observed in LSF water-soluble extracts is consistent with our previous findings in lentils fermented spontaneously or by *L. plantarum* (Torino et al., 2013).

It is noteworthy that the solvent used to extract bioactive compounds is determinant for the content and composition of the compounds extracted. In the present work, water was used as solvent to obtain fermented bean extracts and, therefore, only water-soluble compounds were extracted. Non-fermented water-soluble extracts contained 16-21 mg GAE/g, results that are in agreement with those reported for water soluble extracts from fermented black soybean (Juan & Chou, 2010) and fermented lentils (Torino et al., 2013).

3.4. Phenolic composition of bean extracts

Table 2 and 3 show the phenolic composition of SSF and LSF water-soluble extracts. Several phenolic compounds were identified in non-fermented kidney bean extracts (SSF0). Among them, hydroxycinnamic acids were the major group of phenolic compounds found, representing ~50% of the total content (Table 2). These results are in agreement with those reported by Luthria and Pastor-Corrales (2006) for pinto beans. *p*-Coumaric, ferulic and

sinapic acids linked to aldaric and quinic acids were the hydroxycinnamic derivatives identified. Hydroxybenzoic acids such as *p*-hydroxybenzoic acid, were another group of non-flavonoid compounds identified, representing ~8 % in SSF0 extracts. Among flavonoid compounds, catechins were the most abundant (~23 %), and (+)-catechin and (+)-catechin-hexoside were the main compounds found in SSF0 extracts. Flavanones (~ 13%) such as derivatives of hesperetin, naringenin and eriodictyol, and flavonols (~ 5%) such as glycosides of quercetin and isorhamnetin were also found in SSF0 extracts (Table 2).

Different phenolic compositions were observed in LSF0 extracts (Table 3): catechins (~ 45 %) were the most abundant compounds, followed by hydroxycinnamic derivatives (40 %) and flavanones (9.5 %). Hydroxybenzoic acids (~4 %) and flavonols (~1 %) were the groups present at lower concentrations. LSF0 also contained anthocyanins such as derivatives of cyanidin (cyanidin-3-*O*-glucoside, 2.09±0.15 µg/g), perlargonidin (pelargonidin-3-*O*-glucoside, 14.53±1.13 µg/g; pelargonidin-3-*O*-malonylglucoside, 0.29±0.02 µg/g; and malvidin, 0.96±0.06 µg/g) but these compounds were not found in the SSF0 extracts, likely due to their instability to autoclaving treatment during sample preparation. Some of the phenolic compounds identified in SSF0 and LSF0 extracts have also been found in kidney beans (Lopez et al., 2013).

During SSF and LSF fermentations, qualitative and quantitative differences in the identified phenolic compounds were observed (Table 3). *p*-Hydroxybenzoic acids increased markedly (84 %) in SSF extracts (Table 2) possibly due to their synthesis from bean hemicelluloses by *Bacillus subtilis* (Reddy & Krishnan, 2013), whilst the concentration of these compounds underwent a significant ($P \leq 0.05$) diminution during the LSF process, likely caused by the metabolism p-hydroxybenzoic acids by LAB decarboxylases (Rodriguez, Landete, de las Rivas, & Muñoz, 2008). The highest reduction (~75 %) was found in NF48

359 extracts, with content significantly ($P \leq 0.05$) lower than that observed in LPF48 and LPF96
360 samples.

361 A general increase in the content of hydroxycinnamic compounds was observed in
362 SSF and NF96 extracts. For NF48, LPF48 and LPF96 no significant differences with the
363 corresponding non-fermented extract were found. The content of *trans*-*p*-coumaric acid
364 decreased gradually during SSF (Table 2), whilst it increased during NF and LPF processes,
365 reaching the highest concentration after 96 h (3.44 and 1.48 μg/g in NF and LPF extracts,
366 respectively) (Table 3). The concentration of *trans*-ferulic acid and some of its derivatives
367 increased in SSF and NF extracts (Tables 2 and 3), whilst they decreased significantly ($P \leq$
368 0.05) in LPF samples (Table 3). The novo synthesis of hydroxycinnamic acids such as *p*-
369 coumaric and ferulic acids in fermented legumes has been described previously (Dueñas et al.,
370 2005).

371 (+)-Catechin content underwent a remarkable decrease in all fermented ingredients,
372 reaching levels under the detection limit in SSF samples (Table 3). The decrease of the
373 catechin monomer could be attributed to the formation of more polymeric procyanidins
374 during fermentation, which were not detected in the analytical conditions used. (+)-Catechin-
375 *O*-hexoside was not detected in SSF and LSF ingredients (Tables 2 and 3).

376 No significant differences in the flavanone contents were observed in SSF extracts
377 compared to control (Table 2). However, the concentration of this group of compounds
378 decreased during LSF (45 % in NF96 and 94 % in LPF96) (Table 3).

379 A similar behavior was observed with flavonol concentration, which decreased to 78
380 % in LPF96 extract compared to control (Table 3). These compounds disappeared during SSF
381 (Table 2). A pronounced decrease in flavanones and flavonol content after fermentation of
382 soybean by *B. subtilis* has also been recently reported (Dueñas et al., 2012).

Anthocyanins were not detected in any of the studied extracts, likely due to their degradation during fermentation of kidney beans.

3.5. SDS-PAGE profile of bean extracts

The protein profile of SSF and LSF water-soluble bean extracts is illustrated in Figure 1. SSF0 showed a low number of protein bands in the electropherogram, possibly due to protein denaturation during the autoclaving of cracked seeds prior to fermentation that caused a reduction in protein solubility. A complete absence of protein bands was observed in SSF extracts obtained after 48 and 96 h of fermentation. This effect can be attributed to the severe thermal treatment during autoclaving instead of the hydrolysis of bean proteins by *B. subtilis*. These results match with the low concentration of peptides released during fermentation (Table 1). The lack of protein bands during SDS-PAGE analysis under reducing conditions of natto has also been observed by Weng and Cheng (2010).

Regarding LSF extracts, LSF0 exhibited a complex protein profile, showing protein bands ranging from 6 to 150 kDa, similar to that previously reported for White and Pinto beans (Limón, Peñas, Martínez-Villaluenga, & Frias, 2014; Rui, Boye, Ribereau, Simpson, & Prasher, 2011). The major protein bands showed MW of about 44, 47 and 53 kDa corresponding to individual subunits of vicilin (Rui et al., 2011). Another intense band of ~115 kDa seems to be a large protein or a protein aggregate stabilized by forces other than disulfide bonds, since β-mercaptoethanol was unable to hydrolyze the structure completely. The 31 kDa band probably belongs to phytohemagglutinin, as reported previously by Rui et al. (2011).

During LSF, scarce protein hydrolysis was observed. Only two kidney bean proteins of 95 and 29 kDa were hydrolyzed and peptides with MW $\leq$ 6 kDa (not retained in the gel) were probably formed.

3.6. Antioxidant activity of bean extracts

One of the challenges of legume fermentation is the production of ingredients with enhanced antioxidant activity that may protect against oxidative stress. Antioxidant activity of water-soluble extracts obtained from fermented beans was determined by ORAC-FL assay, one of the most referenced methods to compare antioxidant activity among foods. SSF extracts exhibited the highest antioxidant activity that rose from 170 mg TE/g in SSF0 extracts to 540 and 508 mg TE/g in SSF48 and SSF96 extracts, respectively (Figure 2). These values were somewhat higher than those found in LSF extracts, where only slight significant differences ($P \leq 0.05$) of up to 48 h in NF samples were observed (Figure 2A). These results are higher than those for fermented lentil extracts shown by Torino et al., (2013). These differences indicate that the antioxidant compounds undergo different modifications during legume fermentation that depend not only on the microorganisms involved but also on legume composition.

During fermentation, the bacterial proteolytic activity leads to the release of peptides and to the hydrolysis of phenolic compounds to more simple forms (Lee et al., 2013). Both peptides and soluble phenolic compounds may contribute to the peroxyl-scavenging activity measured by the ORAC-FL method. The low protein hydrolysis observed during SSF in this work suggests that the increase of antioxidant activity in solid fermented kidney beans cannot be attributed to the formation of bioactive peptides during fermentation. This hypothesis is supported by the negative correlation found between ORAC and peptide content ($r = - 0.77$). In contrast, high correlation between ORAC and SPC content was obtained in fermented bean ingredients ($r = 0.94$), suggesting a large contribution of phenolic compounds to the antioxidant activity of these extracts. In this sense, our results are in accordance with those reported by Dueñas et al., (2007), who found a high correlation between specific phenolic

compounds (e.g. *p*-hydroxybenzoic acid and *trans-p*-coumaroylmalic acid) and the antioxidant activity of fermented lentils.

3.7. Angiotensin Converting Enzyme Inhibitory (ACEI) activity of bean extracts

The inhibition of ACE activity by dietary compounds can lead to the reduction of blood pressure and, hence, their consumption may play an important role in promoting cardiovascular health. ACEI activity exerted by the water-soluble extracts obtained in this work is illustrated in Figure 2B. Non-fermented extracts showed a high ACEI activity (>88%), indicating their high antihypertensive potential that can be attributed to their high concentration of phenolic compounds, as shown previously. These results are consistent with those reported by Juan, Wu, and Chou (2010) for black soybean water-soluble extracts.

Fermentation with *B. subtilis* for 48 h led to a sharp decrease in ACEI activity (~34 %), which was maintained up to the end of the SSF process (Figure 2B). These findings indicate that SSF kidney bean extracts could not been considered as antihypertensive ingredients.These results differ substantially from those reported for natto soluble extracts, in which the ACEI activity increased after 18 h fermentation with different *B. subtilis* strains (Juan et al., 2010). The differences found between both studies can be explained by the legume material, the fermentation conditions, as well as the starter strains used during fermentation that probably contribute in a different manner to the formation of ACE inhibitory compounds.

In contrast, the ACEI activity of LSF bean extracts was similar or slightly lower to that observed in unfermented kidney bean extracts (~90 %) (Figure 2B), results that can be attributed to the similar content of SPC (Table 1). The extracts obtained after fermentation for 48h (NF48 and LPF48) were selected to calculate IC_{50} values, since they showed values slightly higher than those obtained at 96h. Both extracts exhibited quite similar IC_{50} values (41.63 and 39.17 µg protein/mL for NF48 and LPF48, respectively). To our knowledge, this

458 is the first study reporting ACEI activity in water-soluble extracts obtained from liquid-state
459 fermented kidney beans. These water-soluble extracts exhibited similar ACEI activity than
460 fermented soybean milk (34.26-39.5 µg protein/mL) (Martinez-Villaluenga et al., 2012) and
461 significantly higher ACEI activity than LSF lentil extracts (200 µg protein/mL) (Torino et al.,
462 2013) and lentil protein hydrolysates (440 µg protein/mL) (Boye, Roufik, Pesta, & Barbana,
463 2010).

464 In order to establish the contribution of the phenolic compounds identified in
465 fermented bean extracts in their antioxidant and ACE inhibitory activities, principal
466 components analysis (PCA) was carried out (Figure 3). Seven components were obtained in
467 both SSF and LSF processes. The first two components accounted for 89.2 % and 99.2 % of
468 the total variance for LSF and SSF, respectively. In both fermentation processes, non-
469 flavonoid compounds (hydroxybenzoic acids and hydroxycinnamic compounds) were
470 positively correlated to antioxidant activity. Previous studies have shown that free and
471 combined hydroxycinnamic compounds exhibit greater antioxidant activity than
472 hydroxybenzoic acids (Alamed, Chaiyasit, McClements, & Decker, 2009). Furthermore, it has
473 been reported that the esterification of *p*-coumaric and ferulic acids leads to the *in vitro*
474 inhibition of low-density lipoprotein oxidation and the esterified compounds show higher
475 protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell
476 culture systems (Kanski, Aksenova, Stoyanova, & Butterfield, 2002). Thus, the dimerization
477 of ferulic acid increased the antioxidant capacity with respect to the free form of
478 hydroxycinnamic acid (Adelakun, Kudanga, Parker, Green, & Roes-Hill, 2012).

479 Flavonoid compounds, catechins and flavonols appeared to be more highly and
480 positively correlated to ACEI activity. Catechins showed the greatest influence on ACEI
481 activity in the case of LSF (Figure 3 A), whilst for SSF the identified flavonols were more
482 highly correlated with ACEI activity (Figure 3 B). Guerrero, Castillo, Quinones, Garcia-

Vallve, Arola, & Pujadas (2012) suggested that flavonoids, due to their structure, are an excellent source of functional antihypertensive products.

4. Conclusions

The results presented in this work reveal that kidney bean var. Pinto is a good source of bioactive compounds and solid and liquid state fermentations are valuable processes to obtain water-soluble functional extracts. SSF extracts presented higher contents of soluble phenolic compounds and antioxidant activity whilst LSF extracts showed potential antihypertensive activity due to their high GABA content and ACEI activity. These findings are of a key relevance not only for the development of water-soluble bean extracts with health-promoting benefits against CVD that can be incorporated as functional ingredients in novel foods and nutraceuticals, but also to encourage the consumption of fermented legumes as a source of bioactive compounds.

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647

Figure captions

Figure 1. SDS-PAGE profiles of water-soluble extracts obtained from kidney beans by solid and liquid state fermentations.

Legend: SSF0, SSF48 and SSF96: water-soluble extracts obtained by solid state fermentation at 0, 48, and 96 h, respectively; LSF0: water-soluble extracts obtained by liquid state fermentation at 0 h; NF48 and NF96: water-soluble extracts obtained by natural fermentation at 48 and 96 h, respectively; LPF48 and LPF96: water-soluble extracts obtained by fermentation with *L. plantarum* at 48 and 96 h, respectively; Mk: Prestained molecular weight marker.

Figure 2. **A)** Antioxidant activity of water-soluble extracts obtained from kidney beans by solid and liquid state fermentations. **B)** ACE inhibitory activity (%) of water-soluble extracts obtained from kidney beans by solid and liquid state fermentations.

Legend: Each value corresponds to the mean of three independent replicates. Different upper-case letters indicate significant differences ($P \leq 0.05$) among solid-state fermentation samples (SSF). Different lower-case letters indicate significant differences ($P \leq 0.05$) among liquid-state fermentation samples (NF, natural fermentation or LPF, fermentation with *L. plantarum*).

Figure 3. Plot of principal components of the ORAC, ACEI and phenolic compounds in water-soluble extracts obtained from kidney beans by **A)** solid-state fermentation (SSF); **B)** liquid-state fermentation (NF, natural fermentation or LPF, fermentation with *L. plantarum*).

Table 1

Table 1. pH values, cell growth, proteolytic activity, GABA and soluble phenolic content in water-soluble extracts obtained from kidney beans by solid and liquid state fermentations.

	Fermentation Time (h)	pH	Log_{10} (CFU/g)*	Peptides (mg/g)	GABA (mg/g)	Soluble phenolic content (mg/g)
SSF	0	6.30 ± 0.07^{a}	5.73 ± 0.03^{a}		3.20 ± 0.14^{a}	15.89 ± 0.56^{a}
	48	6.62 ± 0.12^{b}	8.80 ± 0.12^{b}	5.25 ± 0.43^{a}	2.61 ± 0.18^{b}	31.08 ± 0.86^{b}
	96	7.09 ± 0.03^{c}	8.21 ± 0.15^{b}	29.36 ± 0.68^{b}	2.69 ± 0.23^{b}	35.93 ± 0.69^{c}
NF	0	6.57 ± 0.01^{C}	1.01 ± 0.02^{A}	---	2.27 ± 0.17^{A}	20.68 ± 1.04^{BC}
	48	4.26 ± 0.01^{B}	8.80 ± 0.17^{D}	160.54 ± 14.04^{C}	7.54 ± 0.16^{C}	21.19 ± 0.80^{CD}
	96	4.29 ± 0.01^{B}	8.77 ± 0.13^{D}	113.67 ± 32.62^{B}	10.59 ± 0.52^{E}	20.16 ± 0.62^{B}
LPF	0	6.63 ± 0.03^{C}	7.04 ± 0.11^{C}	---	2.27 ± 0.17^{A}	20.68 ± 1.04^{BC}
	48	3.76 ± 0.12^{A}	9.05 ± 0.05^{E}	157.38 ± 11.85^{C}	6.76 ± 0.28^{B}	21.96 ± 0.54^{D}
	96	3.72 ± 0.03^{A}	5.92 ± 0.03^{B}	98.65 ± 11.11^{A}	9.90 ± 0.10^{D}	17.79 ± 0.08^{A}

Values are means of three independent fermentation batches ± standard deviation. Lower-case superscripts indicate significant differences ($P\leq0.05$) among solid state fermentation samples (SSF). Upper-case superscripts indicate significant differences ($P\leq0.05$) among liquid state fermentation samples (NF, natural fermentation or LPF, fermentation with *L. plantarum*).

*) Log_{10} (CFU/g) for SSF or Log_{10} (CFU/mL) for NF and LPF.

Table 2

Table 2. Concentration of non-anthocyanin phenolic compounds (μg/g) in water-soluble extracts obtained from kidney bean by solid state fermentation.

Compounds	**SSF0**	**SSF48**	**SSF96**
Feruloyl hexoside acid	1.26 ± 0.03[a]	42.42 ± 3.68[b]	40.67±3.07[b]
(+)-Catechin *O*-hexoside	2.76 ± 0.15[b]	nd[a]	nd[a]
Feruloyl aldaric acid	0.29 ± 0.05[a]	8.27 ± 2.02[b]	9.84±0.19[b]
Feruloyl hexoside acid	0.80 ± 0.00[a]	8.60 ± 0.14[b]	8.50±0.00[b]
(+)-Catechin *O*-acylhexoside	nd	nd	nd
Feruloyl aldaric acid	0.79 ± 0.04[a]	15.31 ± 1.41[b]	16.95±0.06[b]
Feruloyl aldaric acid	1.04 ± 0.07[a]	16.57 ± 0.42[b]	33.35±0.61[c]
Sinapoyl aldaric acid	nd	nd	nd
(+)-Catechin	7.88 ± 1.59[b]	nd[a]	nd[a]
Feruloyl aldaric acid	2.72 ± 0.12[a]	31.23 ± 1.93[b]	32.12±3.05[b]
p-Hydroxybenzoic acid	3.48 ± 0.10[a]	20.65 ± 1.45[b]	21.80±1.54[b]
Eriodictyol hexoside	nd[a]	nd[a]	nd[a]
trans-p-Coumaroyl derivative acid	nd[a]	nd[a]	10.73 ± 3.77[b]
trans p-Coumaroyl acylhexoside acid	nd[a]	nd[a]	nd[a]
Sinapoyl methylaldaric acid	nd[a]	nd[a]	nd[a]
Sinapoyl methylaldaric acid	nd[a]	nd[a]	nd[a]
Naringenin derivative	1.26 ± 0.22[a]	1.47 ± 1.24[a]	2.18 ± 0.49[a]
Hesperetin glucuronide-hexoside	3.50 ± 0.05[a]	6.61 ± 1.37[a]	5.14 ± 1.63[a]
trans p-coumaric acid	4.19 ± 0.32[c]	2.40 ± 0.44[b]	0.41 ± 0.23[a]

Naringenin	nd[a]	nd[a]	nd[a]
Ferulic acid	10.56 ± 2.46[a]	63.50 ± 2.82[b]	69.98 ± 13.75[b]
Feruloyl quinic acid	1.36 ± 0.42[c]	nd[a]	nd[a]
Quercetin-3-*O*-glucoside	1.27 ± 0.07b[c]	nd[a]	nd[a]
Quercetin-*O*-acylhexoside	nd[a]	nd[a]	nd[a]
Kaempferol-3-*O*-rutinoside	nd[a]	nd[a]	nd[a]
Eridictyol hexoside	1.35 ± 0.14[b]	nd[a]	nd[a]
Kaempferol-3-*O*-glucoside	0.63 ± 0.01[c]	nd[a]	nd[a]
Quercetin-*O*-hexoside	0.43 ± 0.05[b]	nd[a]	nd[a]
Isorhamnetin-*O*-hexoside	0.03±0.02[a]	nd[a]	nd[a]
Eridictyol-*O*-acyl hexoside	nd[a]	nd[a]	nd[a]
Isorhamnetin-*O*-acyl hexoside	nd[a]	nd[a]	nd[a]
Hydroxybenzoic acids	3.48 ± 0.10[a] (7.62%)	20.65 ± 1.45[b] (9.51%)	21.80 ± 1.54[b] (8.66%)
Hydroxycinnamic compounds	23.03 ± 3.04[a] (50.48%)	188.32 ± 4.33[b] (86.76%)	222.56 ± 9.87[c] (88.43%)
Catechins	10.64 ± 1.74[b] (23.32%)	nd[a]	nd[a]
Flavanones	6.11 ± 0.41[a] (13.39%)	8.08 ± 2.61[a] (3.72%)	7.32 ± 1.14[a] (2.91%)
Flavonols	2.37 ± 0.15[b] (5.18%)	nd[a]	nd[a]
TOTAL	**45.61±1.96**[a]	**217.04±0.28**[b]	**251.68±7.19**[c]

Values are means of three independent fermentation batches ± standard deviation. Lower-case superscripts indicate significant difference ($P \leq 0.05$) among solid state fermentation samples (SSF).

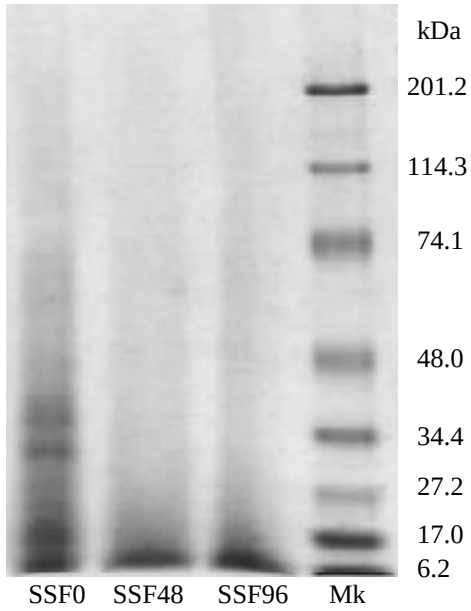
Table 3

Table 3. Concentration of non-anthocyanin phenolic compounds (μg/g) in water-soluble extracts obtained from kidney beans by in liquid state fermentations.

Compounds	LSF0	NF48	NF96	LPF48	LPF96
Feruloyl hexoside acid	4.06 ± 1.36^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
(+)-Catechin *O*-hexoside	14.86 ± 2.69^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Feruloyl aldaric acid	2.31 ± 0.18^{a}	2.54 ± 0.33^{a}	2.25 ± 0.57^{a}	4.50±1.28^{b}	11.95±0.43^{c}
Feruloyl hexoside acid	5.64 ± 0.15^{b}	5.19 ± 0.15^{b}	5.99 ± 0.95^{b}	2.27±0.34^{a}	9.61±0.76^{c}
(+)-Catechin *O*-acylhexoside	23.03 ± 5.31^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Feruloyl aldaric acid	20.84 ± 0.56^{c}	24.99 ± 0.69^{d}	14.11 ± 1.58^{b}	22.26±0.11^{c}	4.63±0.41^{a}
Feruloyl aldaric acid	23.83 ± 0.69^{ab}	23.21 ± 2.77^{ab}	23.14 ± 2.49^{ab}	27.70±8.25^{b}	13.31±1.32^{a}
Sinapoyl aldaric acid	69.41 ± 6.03^{a}	111.18 ± 1.34^{ab}	189.44 ± 38.75^{c}	119.84±2.35^{ab}	147.45±22.93^{bc}
(+)-Catechin	245.54 ± 2.92^{d}	8.54 ± 2.83^{a}	10.07 ± 1.24^{a}	45.78±2.93^{b}	66.93±3.76^{c}
Feruloyl aldaric acid	31.42 ± 0.47^{c}	5.52 ± 0.30^{a}	13.14 ± 0.35^{b}	13.24±0.85^{b}	15.17±2.93^{b}
p-Hydroxybenzoic acid	24.35 ± 0.37^{c}	5.89 ± 1.66^{a}	7.79 ± 0.33^{ab}	9.20±1.80^{b}	9.24±0.57^{b}
Eriodictyol hexoside	12.37 ± 2.35^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
p-Coumaroyl derivative acid	2.53 ± 0.21^{b}	1.37 ± 0.20^{a}	2.55 ± 0.61^{b}	2.34±0.41^{b}	1.06±0.01^{a}
p-Coumaroyl acylhexoside acid	3.08 ± 1.02^{c}	1.41 ± 0.23^{ab}	2.36 ± 0.17^{bc}	0.66±0.09^{a}	0.57±0.20^{a}
Sinapoyl methylaldaric acid	51.57 ± 8.99^{d}	19.73 ± 1.05^{bc}	25.78 ± 6.44^{c}	8.58±0.26^{ab}	5.92±1.31^{a}
Sinapoyl methylaldaric acid	20.84 ± 2.74^{b}	22.64 ± 1.72^{b}	19.92 ± 2.70^{b}	9.98±1.36^{a}	5.32±0.68^{a}
Naringenin derivative	7.48 ± 1.08^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Hesperetin glucuronide-hexoside	20.60 ± 1.56^{d}	17.61 ± 0.92^{c}	17.84 ± 1.13^{c}	7.96±0.84^{b}	3.53±0.52^{a}
trans p-Coumaric acid	nd^{a}	1.47 ± 0.13^{b}	3.44 ± 0.12^{c}	1.29±0.19^{b}	1.48±0.10^{b}

Naringenin	18.04 ± 0.81^{c}	14.78 ± 0.12^{b}	15.30 ± 0.58^{b}	nd^{a}	nd^{a}
Ferulic acid	14.07 ± 0.56^{c}	21.82 ± 0.89^{d}	30.73 ± 2.83^{e}	6.45±1.16^{b}	5.43±0.68^{ab}
Feruloyl quinic acid	nd	nd	nd	nd	nd
Quercetin-3-*O*-glucoside	1.97 ± 0.62^{c}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Quercetin-*O*-acylhexoside	2.55 ± 0.08^{d}	1.18 ± 0.02^{ab}	1.31 ± 0.08^{b}	1.67±0.26^{c}	0.92±0.06^{a}
Kaempferol-3-*O*-rutinoside	0.54 ± 0.00^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Eridictyol-*O*-hexoside	nd	nd	nd	nd	nd
Kaempferol-3-*O*-glucoside	0.34 ± 0.08^{a}	1.57 ± 0.01^{d}	1.83± 0.20^{e}	1.10±0.01^{c}	0.65±0.00^{b}
Quercetin-*O*-hexoside	1.29 ± 0.15^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Isorhamnetin-*O*-hexoside	nd	nd	nd	nd	nd
Eridictyol-*O*-acyl hexoside	0.67 ± 0.05^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Isorhamnetin-*O*-acyl hexoside	0.53± 0.01^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Hydroxybenzoic acids	24.35 ± 0.36^{c} (3.90%)	5.89 ± 1.66^{a} (2.03%)	7.79 ± 0.33^{ab} (2.01%)	9.20 ± 0.60^{b} (3.23%)	9.24 ± 0.60^{b} (3.05%)
Hydroxycinnamic compounds	249.62 ± 4.78^{a} (40.02%)	241.07 ± 3.02^{a} (82.94%)	332.84 ± 44.60^{b} (86.01%)	219.12 ± 14.97^{a} (76.93%)	221.91 ± 31.35^{a} (73.19%)
Catechins	283.43 ± 0.30^{d} (45.44%)	8.54 ± 2.83^{a} (2.94%)	10.07 ± 1.24^{a} (2.60%)	45.78 ± 2.93^{b} (16.07%)	66.93 ± 3.76^{c} (22.08%)
Flavanones	59.15 ± 4.24^{c} (9.48%)	32.39 ± 0.80^{b} (11.14%)	33.14 ± 1.71^{b} (8.56%)	7.96 ± 0.84^{a} (2.79%)	3.53 ± 0.52^{a} (1.16%)
Flavonols	7.22 ± 0.32^{c} (1.16%)	2.76 ± 0.32^{b} (0.95%)	3.14 ± 0.28^{b} (0.81%)	2.77 ± 0.27^{b} (0.97%)	1.57 ± 0.06^{a} (0.52%)
TOTAL	**623.77 ± 8.68^{c}**	**290.65±2.68^{a}**	**368.99±48.15^{b}**	**284.83±14.95^{a}**	**303.19±36.25^{a}**

Values are means of three independent fermentation batches ± standard deviation. Lower-case superscripts indicate significant difference (P≤0.05) among liquid state fermentation samples (NF, natural fermentation or LPF, fermentation with *L. plantarum*).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SSF0
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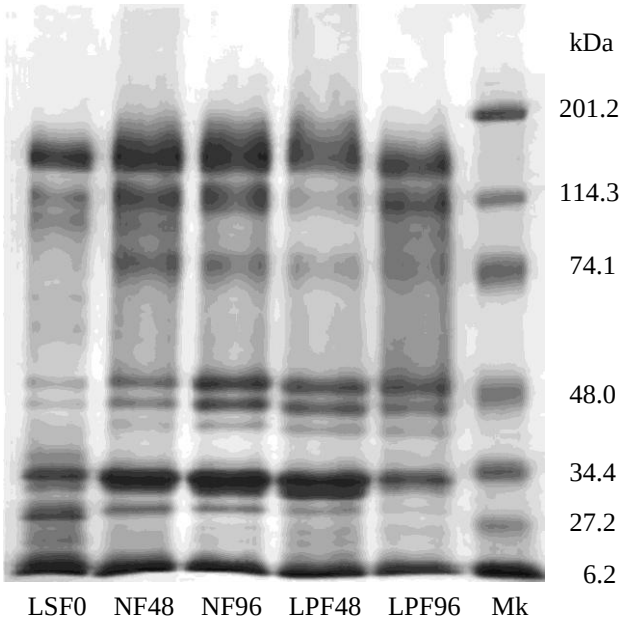
SSF96
Mk


kDa
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Mk

Figure 1

Figure 2

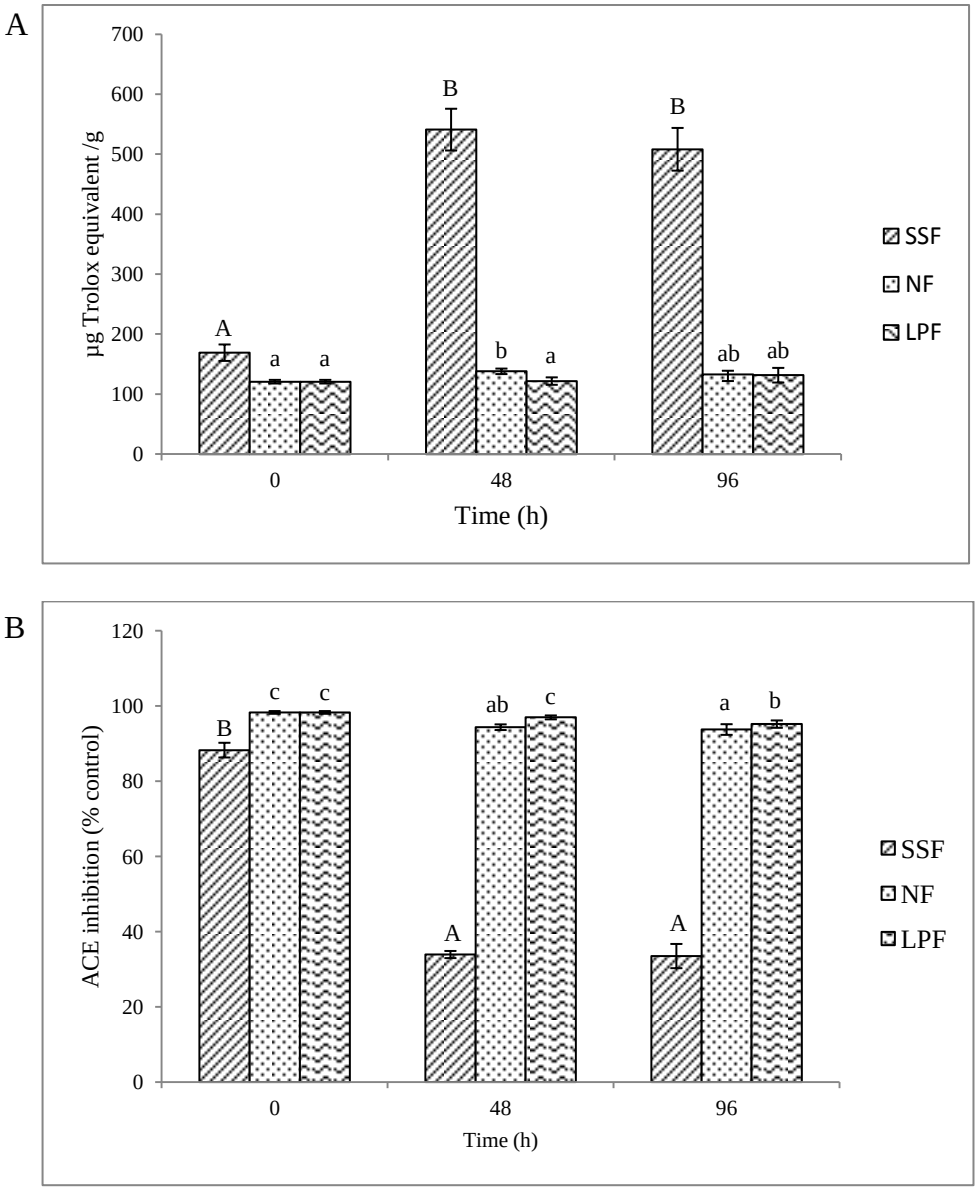

Figure 2

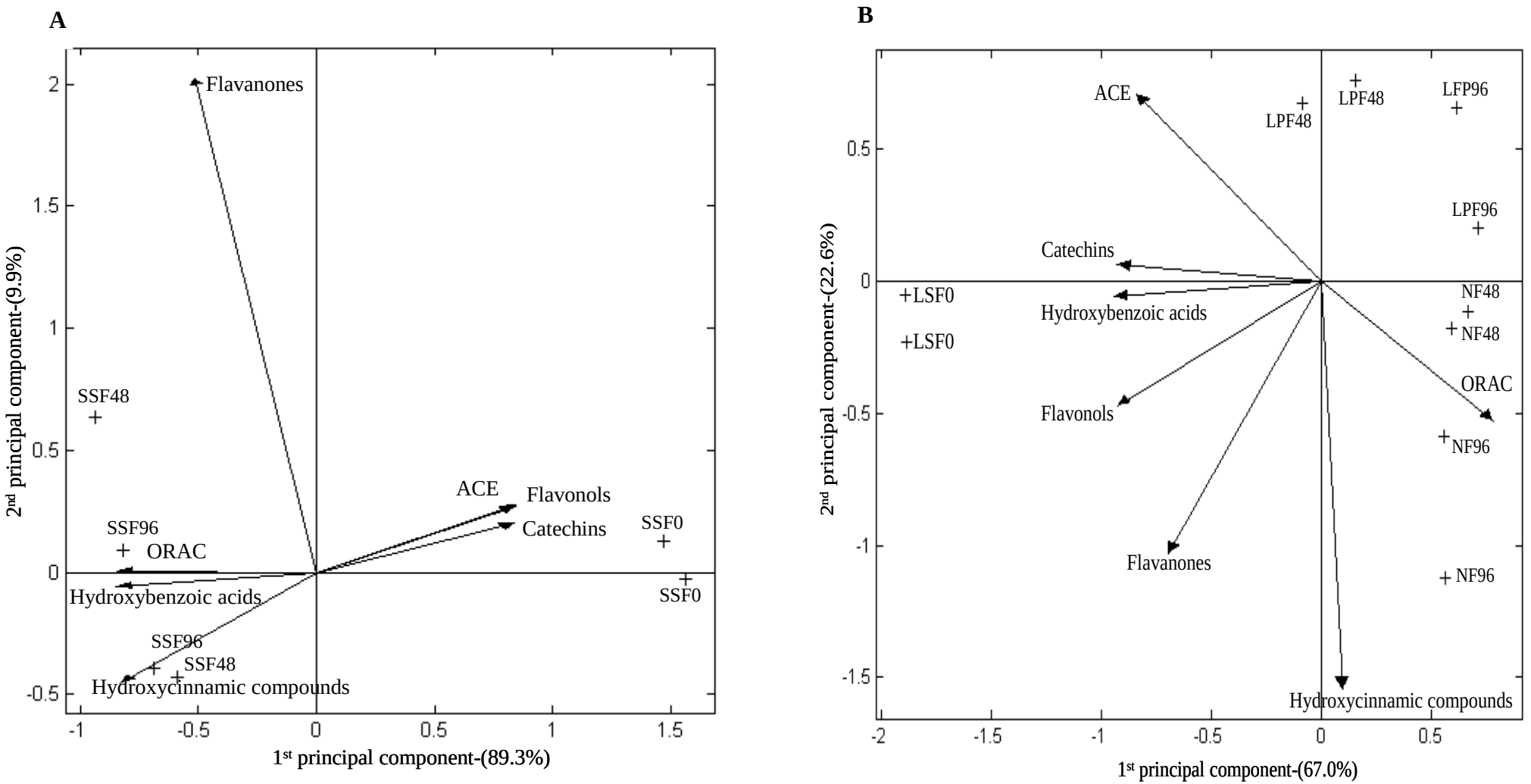
A
Flavanones
SSF48
ACE
Flavonols
Catechins
SSF96
ORAC
SSF0
Hydroxybenzoic acids
SSF96
SSF48
Hydroxycinnamic compounds
2nd principal component-(9.9%)
1st principal component-(89.3%)
B
ACE
LPF48
LPF48
LFP96
LPF96
Catechins
LSF0
Hydroxybenzoic acids
LSF0
NF48
NF48
ORAC
Flavonols
NF96
Flavanones
NF96
Hydroxycinnamic compounds
2nd principal component-(22.6%)
1st principal component-(67.0%)

Figure 3

Madrid 17th June 2014

Dear Editor,

I enclose the manuscript entitled "Fermentation enhances the content of bioactive compounds in kidney bean extracts" that we would like to publish in *Food Chemistry*

Kidney beans contain phytochemicals that provide desirable health benefits beyond basic nutrition by reducing the risk of chronic disease. In this work, we demonstrate the effectiveness of solid and liquid state fermentation processes for the production of bioactive water-soluble extracts. Solid state fermented extracts showed high phenolic content and antioxidant activity, whilst liquid state fermented ones exhibited potential antihypertensive activity due to their high GABA content and ACEI activity. These results highlight fermentation as a valuable process to obtain functional bean ingredients with potential cardioprotective properties.

Authors state that manuscript content has not been published previously, it is not under consideration elsewhere, it is approved by all authors and tacitly or explicitly by the responsible authorities at CSIC, and if accepted, it will not be published elsewhere in English or other language, without the written consent of the copyright-holder.

I am looking forward to hearing from you.

Yours sincerely,

Dr. Juana Frias

Fermentation enhances the content of bioactive compounds in kidney bean extracts

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Abstract

The present work was aimed to evaluate the influence of solid (SSF) and liquid state fermentation (LSF) for 48 and 96 h on the production of functional extracts from kidney beans. SSF was carried out by *Bacillus subtilis*, whilst LSF was performed either spontaneously (NF) or by *Lactobacillus plantarum* strain (LPF). Water-soluble extracts were obtained and the content of γ-aminobutyric acid (GABA) and peptides, the protein profile and the content and composition of soluble phenolic compounds (SPC) were studied. Antioxidant and angiotensin-converting enzyme inhibitory (ACEI) activities were also determined. SSF extracts showed high SPC content (31-36 mg/g) and antioxidant activity (508-541 μg trolox equivalents/g), whilst LSF extracts exhibited potential antihypertensive activity due to their large GABA content (6.8-10.6 mg/g) and ACEI activity (>90 %). Therefore, fermentation can be considered as a valuable process to obtain kidney bean bioactive ingredients, which could encourage their utilization in the formulation of functional foods.

Keywords: kidney beans, fermentation, antioxidants, antihypertensive compounds, functional ingredients.

1. Introduction

Chronic diseases such as cancer, diabetes, obesity and cardiovascular diseases (CVD) are a global health problem and represent the leading cause of mortality in the world, accounting for around 36 billion deaths worldwide each year (WHO, 2013). Scientific evidence has demonstrated that diet has an essential role in their prevention and management.

Consumption of traditional fermented legumes has been shown to have protective effects against CVD (Crujeiras, Parra, Abete & Martinez, 2007) and has, therefore, attracted considerable interest worldwide. Fermentation of legumes brings several advantages since it decreases the non-nutritional factors, improves nutrient digestibility and reduces their allergenicity (Frias, Song, Martinez-Villaluenga, Gonzalez de Mejia, & Vidal-Valverde, 2008; Starzyńska-Janiszewska, Stodolak, & Mickowska, 2013). This technological process also improves the biological activity of legumes, as microbial enzymes bring about the bioconversion of polyphenols into more biologically active compounds (Lee, Lo, & Pan, 2013) and can release bioactive peptides from legume proteins (Martinez-Villaluenga et al., 2012; Torino et al., 2013). Although soybean is the most utilized legume for fermentation, there are other unexplored legumes with a large potential for the production of fermented foods that could contribute to the prevention of CVD.

The type of microorganism used as starter culture plays a key role in the fermentation process. *Bacillus subtilis* has been used as a starter strain for manufacturing soybean fermented products with potential antihypertensive, antithrombotic and fibrinolytic properties (Murakami, Yamanaka, Ohnishi, Fukayama, & Yoshino, 2012; Omura, Hitosugi, Zhu, Ikeda, & Maeda 2005). Recently, Dueñas, Hernández, Robredo, Lamparski, Estrella and Muñoz (2012) have reported that *B. subtilis* enhance the production of isoflavone aglycones, thus improving the antioxidant activity of fermented soybean.

Lactic acid bacteria (LAB) have also been traditionally used for legume fermentation since they are naturally present in legume grains. There is strong consensus that fermentation of legumes with LAB strains, such as those of *Lactobacillus* genera can favor the production of bioactive compounds, providing health benefits beyond basic nutrition (Savijoki, Ingmer, & Varmanen, 2006). Fermentation of cowpeas with *L. plantarum* led to changes in phenolic composition and improved antioxidant activity (Dueñas, Fernández, Hernández, Estrella, & Muñoz, 2005). It has recently been reported that LAB fermentation of adzuki beans and lentils allows accumulation of γ-aminobutyric acid, a blood pressure regulator (Liao, Wang, Shyu, Yu, & Ho, 2013; Torino et al., 2013). In addition, soybean milk fermented with LAB exhibited antioxidant, antihypertensive, antiinflammatory (Martinez-Villaluenga et al., 2012) and anti-obesity properties (Lee et al., 2013).

Kidney beans are attractive commodities due to their nutritional value and phytochemical content. They are mainly consumed in homemade cooked food worldwide whilst their industrial utilization is limited. In accordance with the information mentioned above, the fermentation process could constitute a commercially valuable approach for producing new kidney bean ingredients with health-promoting properties to prevent CVD.

Therefore, the challenge of this work was to explore solid and liquid state fermentations in kidney beans for the production of water-soluble extracts with potential antihypertensive effects (angiotensin I converting enzyme inhibitory activity and GABA content), as well as high content of bioactive phenolic compounds and antioxidant activity. The evolution of microbial populations and pH in fermented beans was also studied. Results derived from this work could provide information on the functional properties of kidney bean fermented ingredients that may enhance the industrial utilization of this legume crop for novel food applications.

2. Materials and methods

2.1. Seeds

Kidney beans (*Phaseolus vulgaris* var. Pinto) were provided by Semillas Iglesias S.A. (Salamanca, Spain). Seeds were washed with distilled water and dried at 55 °C for 24 h before fermentation.

2.2. Preparation of microbial cultures

Bacillus subtilis CECT 39^T (ATCC 6051) and *Lactobacillus plantarum* CECT 748^T (ATCC 14917) were purchased from the Spanish Type Culture Collection (CECT). Cultures were stored and activated for solid or liquid fermentations as described in Torino et al. (2013).

2.3. Fermentation of kidney beans

2.3.1. Solid state fermentation (SSF)

Cracked beans (100 g) were suspended in sterile distilled water (1:2, w/v) and autoclaved at 121 ºC for 15 min. Sterile cracked seeds (30 g) were homogeneously inoculated with 5 % (v/w) *B. subtilis* (10^5 CFU/g), mixed, aseptically distributed over Petri dishes and incubated for 96 h at 30 ºC and 90 % humidity in a climatic chamber (Snijders-Scientific, Tiburg, Netherlands). SSF samples were withdrawn at 0, 48 and 96 h to determine changes in bacterial populations and pH values. Fermented samples were autoclaved at 121 ºC for 15 min and freeze-dried. SSF water-soluble extracts were prepared by suspending freeze-dried fermented beans in distilled water (1:10, w/v) and kept in continuous agitation for 1 h at room temperature. Extracts were centrifuged at 14534 *g* for 15 min at 10 ºC. Supernatants were filtered through Whatman nº 1 paper, freeze-dried and stored under vacuum at -20 ºC until further analysis.

2.3.2. Liquid state fermentations (LSF).

Bean flour (200 g) was suspended in sterile distilled water (1:5 w/v). Fermentations were carried out in a 3 L BioFlo/celligen 115 fermenter (New Brunswick Scientific Co., INC, Edison, NJ) either spontaneously by the indigenous microbiota present on seeds (natural fermentation, NF) or with 2 % (v/v) of *L. plantarum* suspension (10^8 CFU/mL) (LPF), at 37 ºC and continuous agitation at 350 rpm for 96 h. Samples were withdrawn at 0, 48 and 96 h to determine changes in bacterial populations and pH. Fermented samples were centrifuged (14534 *g*, 15 min, 6 ºC) and supernatants were freeze-dried and stored under vacuum at -20 ºC for further analysis.

2.4. Microbiological analysis

Viable cells of *B. subtilis* were enumerated in BHI broth supplemented with 1.5 % (w/v) agar, after incubation at 30 ºC for 48 h. LAB were enumerated in MRS agar after incubation at 37 ºC in a 5 % CO_2 atmosphere for 72 h. Cell counts were expressed as $\log_{10}$ CFU/g for *B. subtilis* and $\log_{10}$ CFU/mL for LAB.

2.5. Chemical analysis

2.5.1. Proteolytic activity

Proteolytic activity in SSF and LSF bean extracts was assessed by measuring the free amino groups following the method reported by Martinez-Villaluenga et al. (2012). Results were expressed as mg peptides/g extract.

2.5.2. GABA content.

The extraction of GABA in SSF and LSF extracts and its quantification by HPLC was carried out as in Torino et al. (2013). Results were expressed in mg GABA/g extract.

2.5.3. Soluble phenolic compounds (SPC)

The content of SPC in bean extracts was determined as in Torino et al., (2013). SPC were quantified by external calibration using gallic acid (Sigma-Aldrich). Results were expressed as mg gallic acid equivalents (GAE)/g.

2.5.4. Extraction and analysis of phenolic compounds by HPLC-DAD-ESI/MS

Water-soluble bean extracts were macerated in methanol/trifluoroacetic acid ($1^0/_{000}$):water 80:20 (v/v) for 1 h according to Dueñas, Hernandez, & Estrella (2007) and analysed as follows:

Non anthocyanin phenolic compounds. The extracts were analyzed using a Hewlett-Packard 1100MS chromatograph (Agilent Technologies) following the method described by Barros, Dueñas, Carvalho, Ferreira, & Santos-Buelga (2012). MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer controlled by the Analyst 5.1 software as in Barros et al. (2012).

Anthocyanins. The extracts were analysed in an AQUA® HPLC system (Phenomenex) equipped with a reverse phase C18 column (5 μm, 150 mm × 4.6 mm i.d) at 35 ºC according to Garcia-Marino, Hernández-Hierro. Rivas-Gonzalo, and Escribano-Bailón (2010). Detection was carried out at 520 nm. MS was performed in the same equipment described above. Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 ºC) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high). Both quadrupol units were set at unit resolution. The ion spray voltage was set at 5000V in the positive mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively. Setting parameters used for EMS mode were: declustering potential (DP) 41 V, entrance potential (EP) 7.5 V, collision energy (CE) 10 V, and

parameters for EPI mode were: DP 41 V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V.

2.5.5. SDS-PAGE protein profile

Proteins from SSF and LSF extracts were separated by SDS-PAGE under reducing conditions according to Limón, Peñas, Martinez-Villaluenga, and Frias (2014).

2.5.6. Antioxidant activity

Oxygen Radical Absorbance Capacity was determined in the water-soluble bean extracts by fluorescence measurement (ORAC-FL) as described by Torino et al., (2013). Trolox (Sigma) was used as standard. Results were expressed as µg Trolox equivalents (TE)/g extract.

2.5.7. Angiotensin converting enzyme inhibitory activity (ACEI activity)

ACEI activity was determined according to Martínez-Villaluenga et al., (2012). IC_{50} values, corresponding to the amount of sample that inhibits the activity of ACE by 50% were determined by sigmoidal dose–response curves in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

2.6. Statistical methods. SSF and LSF processes were performed in triplicate, chemical analyses were carried out in duplicate, and each determination was analyzed three times. Data were subjected to one-way analysis of variance (ANOVA) using Statgraphics Centurion XVI software, version 16.1.17 (Statistical Graphics Corp, Rockville, MD, USA). Significant differences between samples were determined by using the least significant difference test at $P \leq 0.05$ probability level.

3. Results and discussion

3.1. Evolution of pH and microbial growth during fermentation

Table 1 shows the changes of pH values during fermentation of beans at the different studied conditions. During SSF pH value significantly increased from 6.3 to 7.0, which could be attributed to the production of ammonia during kidney bean fermentation by *B. subtilis*. The population of *B. subtilis* increased sharply during the first 48 h (~3.14 log units) and no further changes were observed. Bacterial growth was reflected in the proteolytic activity, measured as the amount of peptides released during fermentation, which increased significantly from 48 h (5.25 mg/g) to 96 h (29.36 mg/g) (Table 1). The low amount of peptides formed during the first 48 h could be caused by the difficulty of *B. subtilis* proteases to cleave glycoproteins, phosphoproteins or domains containing a high number of disulfide bridges (Weng & Chen, 2010).

In the course of LSF, pH values gradually decreased from 6.6 to 4.3 and 3.7 in NF96 and LPF96, respectively (Table 1) due to the production of organic acids by LAB during fermentation. The initial population of LAB in raw beans was very low (1.01 CFU/mL) and it increased significantly during NF, reaching counts of 8.8 log CFU/mL after 48 h, which remained almost constant to the end of the fermentation (Table 1). LAB counts increased from 7.0 to 9.1 log CFU/mL after 48 h and suffered a significant ($P \leq 0.05$) reduction at the end of fermentation (5.9 log CFU/mL). When colonies from MRS plates were subjected to microscopy analysis, bacilli morphologically corresponding to *L. plantarum* were observed, indicating that the starter added led the fermentation process. The loss of viability of *L. plantarum* after 96 h was previously observed by our group during induced lentil fermentation (Torino et al., 2013). It is known that organic acids released during fermentation exert an inhibitory effect on microbial growth due to an increase in the amount of protons that

211 acidifies the cytoplasm and inhibits many metabolic functions (Lambert & Stratford, 1999). It
212 has been shown that the growth of *L. plantarum* is inhibited when internal pH reach values of
213 4.6-4.8, corresponding to external cellular pH values of ~3 (McDonald, Fleming, & Hassan,
214 1990). These findings could explain the loss of viability of *L. plantarum* observed in our
215 study after 96 h (pH 3.7).

216 LAB counts were consistent with pH values in NF and LPF. Faster acidification was
217 observed in LPF compared to NF due to the higher LAB population. Growth of the LAB
218 population in LSF after 48h was accompanied by a noticeable increase in the proteolytic
219 activity and, as a result, significantly higher concentrations of peptides were found (~160
220 mg/g extract) (Table 1). However, peptide content was significantly reduced ($P \leq 0.05$) after 96
221 h in both LSF. This effect could be attributed to the microbial utilization of peptides as a
222 nitrogen source.

223

224 *3.2. GABA content in bean extracts*

225 GABA contents in water-soluble extracts are also compiled in Table 1. SSF led to a
226 decrease in GABA content (~18%) irrespective of fermentation time. Unlike SSF, NF and
227 LPF processes caused a significant ($P \leq 0.05$) GABA accumulation. The extent of this increase
228 depended on the type of fermentation: NF led to significantly ($P \leq 0.05$) higher GABA levels
229 than LPF (10.6 vs. 9.9 mg/g for NF96 and LPF96, respectively). Similar GABA accumulation
230 was reported in adzuki bean milk fermented with *Lactococcus lactis* and *Lactobacillus*
231 *rhamnosus* (Liao et al., 2013). These results indicate that effects of fermentation on GABA
232 content are due to the type of microorganism involved in the process. GABA is a non-protein
233 amino acid produced from L-glutamic acid by glutamic acid decarboxylase enzyme (GAD).
234 *B. subtilis* seems to have a weak capacity to produce GABA, mostly when compared with
235 other microorganisms such as LAB (Park & Oh, 2006). These findings are in agreement with

the low GABA content observed in SSF bean extracts in this work. In addition, the thermal treatment of cracked seeds during autoclaving used in the SSF process would inactivate endogenous GAD activity from beans, contributing to the reduced GABA concentration observed in SSF extracts. On the contrary, the GABA accumulation observed in LSF (Table 1) suggests that LAB strains stimulate the production of GABA during bean fermentation. It has been previously shown that many LAB strains, including *L. plantarum*, are potential GABA producers (Di Cagno et al., 2010), since they harbor the gene encoding GAD enzyme (Ko, Lin, & Tsai, 2013). It can be postulated that the endogenous bean GAD would be in part responsible for the GABA increase observed during LSF, since previous studies have revealed a rapid plant GAD activation in response to mechanical manipulation, anaerobic conditions and low pH (Akihiro et al., 2008). The higher GABA content found in NF compared to LPF extracts could be attributed to the higher number of LAB strains producing GAD in NF and also to the different pH of both fermentations. pH seems to play a crucial role in GABA production by LAB. It has been reported that *L. plantarum* DSM19463 synthesized the maximum GABA concentration at pH 6.0 (Di Cagno et al., 2010). The acidic pH observed in LSF in this work could be one of the causes of the decreased GAD activity and, consequently, the low rate of GABA production.

It is well known that GABA has a blood pressure-lowering effect in animals and humans (Inoue et al., 2003) and also acts as a strong secretagogue of insulin from the pancreas, potentially preventing diabetes (Adeghate & Ponery, 2002). Therefore, there is a growing interest to develop GABA-enriched functional foods and fermentation of beans by LAB could constitute an attractive approach to achieve this purpose.

3.3. Content of soluble phenolic compounds in bean extracts (SPC)

Due to the potential positive effects of phenolic compounds and their metabolites, they are the subject of many ongoing research studies. Polyphenols are natural antioxidants, which may protect against multiple chronic diseases (Del Rio, Rodriguez-Mateos, Spencer, Tognolini, Borges, & Crozier, 2013) and legumes are considered a rich source of these compounds. SPC concentration varied in fermented beans depending on the fermentation conditions (Table 1). SSF resulted in a significant ($P \leq 0.05$) increase in SPC after 48 and 96 h (96 and 126 %, respectively), whilst no significant ($P \geq 0.05$) changes or a slight decrease was observed at the end of LSF fermentations. The increase in SPC after SSF could be related to the production of β-glucosidases by *B. subtilis*. This enzyme hydrolyzes β-glucosidic bonds of several phenolic compounds occurring in a conjugated form (phenolic glucosides) to the corresponding phenolic aglycones, increasing the concentration of free polyphenols (Georgetti, Vicentini, Yokoyama, Borin, Spadaro, & Fonseca, 2009). In fact, the SPC content observed after SSF is within the range of values reported after fermentation of soybean by *B. subtilis* TN51 (Dajanta, Apichartsrangkoon, & Chukeatirote, 2011). Our results indicate that microbial β-glucosidase activity was higher in SSF than in the LSF process. These differences could be attributed to the different fermentation conditions. Sestelo, Poza, and Villa, (2004) reported that the activity of β-glucosidase produced by *L. plantarum* USCI varied greatly depending on the pH in the medium, with the optimal pH range for enzyme activity being from 4.5 to 7.5, while pH values below 4 produced enzyme inactivation. These findings could explain the low modification of SPC content during LSF, due to inactivation of this enzyme at low pH values (3.7). The lack of changes in SPC observed in LSF water-soluble extracts is consistent with our previous findings in lentils fermented spontaneously or by *L. plantarum* (Torino et al., 2013).

It is noteworthy that the solvent used to extract bioactive compounds is determinant for the content and composition of the compounds extracted. In the present work, water was

used as solvent to obtain fermented bean extracts and, therefore, only water-soluble compounds were extracted. Non-fermented water-soluble extracts contained 16-21 mg GAE/g, showing water as an efficient solvent for extracting phenolic compounds. The SPC content in water-soluble extracts obtained from fermented beans is in agreement with those reported for water soluble extracts from fermented black soybean (Juan & Chou, 2010) and fermented lentils (Torino et al., 2013).

3.4. Phenolic composition of bean extracts

Table 2 and 3 show the phenolic composition of SSF and LSF water-soluble extracts. Several phenolic compounds were identified in non-fermented kidney bean extracts (SSF0). Among them, hydroxycinnamic acids were the major group of phenolic compounds found, representing ~50% of the total content (Table 2). These results are in agreement with those reported by Luthria and Pastor-Corrales (2006) for pinto beans. *p*-Coumaric, ferulic and sinapic acids linked to aldaric and quinic acids were the hydroxycinnamic derivatives identified. Hydroxybenzoic acids such as *p*-hydroxybenzoic acid, were another group of non-flavonoid compounds identified, representing ~8 % in SSF0 extracts. Among flavonoid compounds, catechins were the most abundant (~23 %), and (+)-catechin and (+)-catechin-hexoside were the main compounds found in SSF0 extracts. Flavanones (~ 13%) such as derivatives of hesperetin, naringenin and eriodictyol, and flavonols (~ 5%) such as glycosides of quercetin and isorhamnetin were also found in SSF0 extracts (Table 2).

Different phenolic compositions were observed in LSF0 extracts (Table 3): catechins (~ 45 %) were the most abundant compounds, followed by hydroxycinnamic derivatives (40 %) and flavanones (9.5 %). Hydroxybenzoic acids (~4 %) and flavonols (~1 %) were the groups present at lower concentrations. LSF0 also contained anthocyanins such as derivatives of cyanidin (cyanidin-3-*O*-glucoside, 2.09±0.15 μg/g), perlargonidin (pelargonidin-3-*O*-

glucoside, 14.53±1.13 μg/g; pelargonidin-3-*O*-malonylglucoside, 0.29±0.02 μg/g; and malvidin, 0.96±0.06 μg/g) but these compounds were not found in the SSF0 extracts. Some of the phenolic compounds identified in SSF0 and LSF0 extracts have also been found in kidney beans (Lopez et al., 2013).

During SSF and LSF fermentations, qualitative and quantitative differences in the identified phenolic compounds were observed. *p*-Hydroxybenzoic acid increased markedly (84 %) in SSF extracts (Table 2), whilst the concentration of this compound underwent a significant ($P \leq 0.05$) diminution during the LSF process (Table 3). The highest reduction (~75 %) was found in NF48 extracts, with content significantly ($P \leq 0.05$) lower than that observed in LPF48 and LPF96 samples.

A general increase in the content of hydroxycinnamic compounds was observed in SSF and NF96 extracts. For NF48, LPF48 and LPF96 no significant differences with the corresponding non-fermented extract were found. The content of *trans*-*p*-coumaric acid decreased gradually during SSF (Table 2), whilst it increased during NF and LPF processes, reaching the highest concentration after 96 h (3.44 and 1.48 μg/g in NF and LPF extracts, respectively) (Table 3). The concentration of *trans*-ferulic acid and some of its derivatives increased in SSF and NF extracts (Tables 2 and 3), whilst they decreased significantly ($P \leq 0.05$) in LPF samples (Table 3). The novo synthesis of hydroxycinnamic acids such as *p*-coumaric and ferulic acids in fermented legumes has been described previously (Dueñas et al., 2005).

(+)-Catechin content underwent a remarkable decrease in all fermented ingredients, reaching levels under the detection limit in SSF samples (Table 3). The decrease of the catechin monomer could be attributed to the formation of more polymeric procyanidins during fermentation, which were not detected in the analytical conditions used. (+)-Catechin-*O*-hexoside was not detected in SSF and LSF ingredients (Tables 2 and 3).

No significant differences in the flavanone contents were observed in SSF extracts compared to control (Table 2). However, the concentration of this group of compounds decreased during LSF (45 % in NF96 and 94 % in LPF96) (Table 3).

A similar behavior was observed with flavonol concentration, which decreased to 78 % in LPF96 extract compared to control (Table 3). These compounds disappeared during SSF (Table 2). A pronounced decrease in flavanones and flavonol content after fermentation of soybean by *B. subtilis* has also been recently reported (Dueñas et al., 2012).

Anthocyanins were not detected in any of the fermented extracts studied.

3.5. SDS-PAGE profile of bean extracts

The protein profile of SSF and LSF water-soluble bean extracts is illustrated in Figure 1. SSF0 showed a low number of protein bands in the electropherogram, possibly due to protein denaturation during the autoclaving of cracked seeds prior to fermentation that caused a reduction in protein solubility. A complete absence of protein bands was observed in SSF extracts obtained after 48 and 96 h of fermentation. This effect can be attributed to the severe thermal treatment during autoclaving instead of the hydrolysis of bean proteins by *B. subtilis*. These results match with the low concentration of peptides released during fermentation (Table 1). The lack of protein bands during SDS-PAGE analysis under reducing conditions of natto has also been observed by Weng and Cheng (2010).

Regarding LSF extracts, LSF0 exhibited a complex protein profile, showing protein bands ranging from 6 to 150 kDa, similar to that previously reported for White and Pinto beans (Limón, Peñas, Martínez-Villaluenga, & Frias, 2014; Rui, Boye, Ribereau, Simpson, & Prasher, 2011). The major protein bands showed MW of about 44, 47 and 53 kDa corresponding to individual subunits of vicilin (Rui et al., 2011). Another intense band of ~115 kDa seems to be a large protein or a protein aggregate stabilized by forces other than

disulfide bonds, since β-mercaptoethanol was unable to hydrolyze the structure completely. The 31 kDa band probably belongs to phytohemagglutinin, as reported previously by Rui et al. (2011).

During LSF, scarce protein hydrolysis was observed. Only two kidney bean proteins of 95 and 29 kDa were hydrolyzed and peptides with MW ≤ 6 kDa (not retained in the gel) were probably formed.

3.6. Antioxidant activity of bean extracts

One of the challenges of legume fermentation is the production of ingredients with enhanced antioxidant activity that may protect against oxidative stress. Antioxidant activity of water-soluble extracts obtained from fermented beans was determined by ORAC-FL assay, one of the most referenced methods to compare antioxidant activity among foods. SSF extracts exhibited the highest antioxidant activity that rose from 170 mg TE/g in SSF0 extracts to 540 and 508 mg TE/g in SSF48 and SSF96 extracts, respectively (Figure 2). These values were somewhat higher than those found in LSF extracts, where only slight significant differences ($P \leq 0.05$) of up to 48 h in NF samples were observed (Figure 2A). These results are higher than those for fermented lentil extracts shown by Torino et al., (2013). These differences indicate that the antioxidant compounds undergo different modifications during legume fermentation that depend not only on the microorganisms involved but also on legume composition.

During fermentation, the bacterial proteolytic activity leads to the release of peptides and to the hydrolysis of phenolic compounds to more simple forms (Lee et al., 2013). Both peptides and soluble phenolic compounds may contribute to the peroxyl-scavenging activity measured by the ORAC-FL method. The low protein hydrolysis observed during SSF in this work suggests that the increase of antioxidant activity in solid fermented kidney beans cannot

be attributed to the formation of bioactive peptides during fermentation. This hypothesis is supported by the negative correlation found between ORAC and peptide content (r=-0.77). In contrast, high correlation between ORAC and SPC content was obtained in fermented bean ingredients (r=0.94), showing the high contribution of phenolic compounds to the antioxidant activity of these extracts. In this sense, our results are in accordance with those reported by Dueñas et al., (2007), who found a high correlation between specific phenolic compounds (e.g. *p*-hydroxybenzoic acid and *trans*-*p*-coumaroylmalic acid) and the antioxidant activity of fermented lentils.

3.7. Angiotensin Converting Enzyme Inhibitory (ACEI) activity of bean extracts

The inhibition of ACE activity by dietary compounds can lead to the reduction of blood pressure and, hence, their consumption may play an important role in promoting cardiovascular health. ACEI activity exerted by the water-soluble extracts obtained in this work is illustrated in Figure 2B. Non-fermented extracts showed a high ACEI activity (>88%), indicating their high antihypertensive potential that can be attributed their high concentration of phenolic compounds, as shown previously. These results are consistent with those reported by Juan, Wu, and Chou (2010) for black soybean water-soluble extracts.

Fermentation with *B. subtilis* for 48 h led to a sharp decrease in ACEI activity (~34 %), which was maintained up to the end of the SSF process (Figure 2B). These findings indicate that SSF kidney bean extracts could not been considered as antihypertensive ingredients.These results differ substantially from those reported for natto soluble extracts, in which the ACEI activity increased after 18 h fermentation with different *B. subtilis* strains (Juan et al., 2010). The differences found between both studies can be explained by the legume material, the fermentation conditions, as well as the starter strains used during

409 fermentation that probably contribute in a different manner to the formation of ACE
410 inhibitory compounds.

411 In contrast, the ACEI activity of LSF bean extracts was similar or slightly lower to that
412 observed in unfermented kidney bean extracts (~90 %)(Figure 2B), results that can be
413 attributed to the similar content of SPC (Table 1). The extracts obtained after fermentation for
414 48h (NF48 and LPF48) were selected to calculate IC_{50} values, since they showed values
415 slightly higher than those obtained at 96h. Both extracts exhibited quite similar IC_{50} values
416 (41.63 and 39.17 µg protein/mL for NF48 and LPF48, respectively). To our knowledge, this
417 is the first study reporting ACEI activity in water-soluble extracts obtained from liquid-state
418 fermented kidney beans. These water-soluble extracts exhibited similar ACEI activity than
419 fermented soybean milk (34.26-39.5 µg protein/mL) (Martinez-Villaluenga et al., 2012) and
420 significantly higher ACEI activity than LSF lentil extracts (200 µg protein/mL) (Torino et al.,
421 2013) and lentil protein hydrolysates (440 µg protein/mL) (Boye, Roufik, Pesta, & Barbana,
422 2010).

423 In order to establish the contribution of the phenolic compounds identified in
424 fermented bean extracts in their antioxidant and ACE inhibitory activities, principal
425 components analysis (PCA) was carried out (Figure 3). Seven components were obtained in
426 both SSF and LSF processes. The first two ones accounted for 89.2 % for LSF and 99.2 % for
427 SSF of the total variance. In both fermentation processes, non-flavonoid compounds
428 (hydroxybenzoic acids and hydroxycinnamic compounds) were positively correlated to
429 antioxidant activity. Previous studies have shown that free and combined hydroxycinnamic
430 compounds exhibit greater antioxidant activity than hydroxybenzoic acids (Alamed,
431 Chaiyasit, McClements, & Decker, 2009). Furthermore, it has been reported that the
432 esterification of *p*-coumaric and ferulic acids leads to the *in vitro* inhibition of low-density
433 lipoprotein oxidation and the esterified compounds show higher protection against hydroxyl

and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems (Kanski, Aksenova, Stoyanova, & Butterfield, 2002). Thus, the dimerization of ferulic acid increased the antioxidant capacity with respect to the free form of hydroxycinnamic acid (Adelakun, Kudanga, Parker, Green, & Roes-Hill, 2012).

Flavonoid compounds, catechins and flavonols appeared to be more highly and positively correlated to ACEI activity. Catechins showed the greatest influence on ACEI activity in the case of LSF (Figure 3 A), whilst for SSF the identified flavonols were more highly correlated with ACEI activity (Figure 3 B). Guerrero, Castillo, Quinones, Garcia-Vallve, Arola, & Pujadas (2012) suggested that flavonoids, due to their structure, are an excellent source of functional antihypertensive products.

4. Conclusions

The results presented in this work reveal that kidney bean var. Pinto is a good source of bioactive compounds and solid and liquid state fermentations are valuable processes to obtain water-soluble functional extracts. SSF extracts presented higher contents of soluble phenolic compounds and antioxidant activity whilst LSF extracts showed potential antihypertensive activity due to their high GABA content and ACEI activity. These findings are of relevance not only for the development of water-soluble bean extracts with health-promoting benefits against CVD that can be incorporated as functional ingredients in novel foods and nutraceuticals, but also to encourage the consumption of fermented legumes as a source of bioactive compounds.

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Figure captions

Figure 1. SDS-PAGE profiles of water-soluble extracts obtained from kidney beans by solid and liquid state fermentations.

Legend: SSF0, SSF48 and SSF96: water-soluble extracts obtained by solid state fermentation at 0, 48, and 96 h, respectively; LSF0: water-soluble extracts obtained by liquid state fermentation at 0 h; NF48 and NF96: water-soluble extracts obtained by natural fermentation at 48 and 96 h, respectively; LPF48 and LPF96: water-soluble extracts obtained by fermentation with *L. plantarum* at 48 and 96 h, respectively; Mk: Prestained molecular weight marker.

Figure 2. **A)** Antioxidant activity of water-soluble extracts obtained from kidney beans by solid and liquid state fermentations. **B)** ACE inhibitory activity (%) of water-soluble extracts obtained from kidney beans by solid and liquid state fermentations.

Legend: Each value corresponds to the mean of three independent replicates. Different upper-case letters indicate significant differences ($P \leq 0.05$) among solid-state fermentation samples (SSF). Different lower-case letters indicate significant differences ($P \leq 0.05$) among liquid-state fermentation samples (NF, natural fermentation or LPF, fermentation with *L. plantarum*).

Figure 3. Plot of principal components of the ORAC, ACEI and phenolic compounds in water-soluble extracts obtained from kidney beans by **A)** solid-state fermentation (SSF); **B)** liquid-state fermentation (NF, natural fermentation or LPF, fermentation with *L. plantarum*).